Functional genomics: Gene identification via T-DNA mediated gene trap tagging in plants

TANG Wei, Vanessa Samuels, Janet Ogbon, Aquilla McCoy
(North Carolina State University, Department of Forestry, Forest Biotechnology Group, Centennial Campus Box 7247, Raleigh, NC 27695-7247, USA)

Abstract: The fully sequenced genomes of Arabidopsis, rice, tomato, potato, maize, wheat, and soybean offer large amounts of information about cellular and developmental biology. It is a central challenge of genomics to use this information in discovering the function of proteins and identifying developmentally important genes. Although classical genetic approaches to gene identification which rely on disruption of a gene leading to a recognizable phenotype continues to be an extremely successful one, T-DNA mediated gene trap tagging which has been developed that utilize random integration of reporter gene constructs has also proven to be an extremely powerful tool in plant cellular developmental biology. In this review, how gene trap tagging, promoter trap tagging, and enhancer trap tagging detection systems have been applied to plant biology is described and these gene identification techniques could be useful to the plant molecular biology and plant biotechnology community.
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Introduction

Since a technique that allows gene activity to be monitored by creating gene fusions with a reporter gene was first used in bacterial genetics 20 years ago (Casadaban and Cohen 1979), random insertions of a reporter gene into the *Escherichia coli* chromosome could be used to monitor the expression of individual genes. In this way, genes could be identified based on their pattern of expression over time or under a variety of conditions in the absence of a mutant phenotype or any sequence information. Because the sequence of the inserted DNA was known, it provided a "tag" for easy isolation of the chromosomal gene. This approach, called gene trap, has been modified for use in a number of species and has been extensively exploited in animal and plant species (Bellen 1999). Gene traps provide a powerful tool for gene identification. Genes are identified based on reporter gene expression; therefore, a mutant phenotype is not required. This advantage allows identification of two classes of genes that are not easily amenable to classic genetic analysis: functionally redundant genes and genes that have functions at multiple developmental stages. Gene traps allow genes to be identified based solely on expression pattern, so that loss-of-function mutations are not mandatory. Although a gene trap insertion may disrupt gene function, the disruption is not necessary for gene identification. Therefore, functionally redundant genes whose expression patterns meet screening criteria can be identified in the absence of an easily recognizable phenotype. Moreover, essential genes can be identified based on reporter gene expression.

For research on gene trap tagging, a collection of individuals that contains randomly integrated reporter gene into the genome must be generated. Various strategies for obtaining efficient reporter gene expression have been utilized. Each insertion is maintained as a separate line, which can subsequently be screened for reporter gene expression. Screens can be designed to identify genes that are expressed in specific cells or tissues, at specific developmental stages, or in response to an environmental stimulus. Reporter genes can be used to construct three basic types of gene trap: gene trap tagging, promoter trap tagging, and enhancer trap tagging (Fig. 1A-D). Each type is able to respond to *cis*-acting regulatory sequences at the site of insertion. Gene traps and promoter traps contain a promoterless reporter gene so that expression can occur only when the insertion is within a transcriptional unit and in the correct orientation (Fig. 1B and 1C). Expression of a promoter trap reporter gene requires that it be inserted into an exon, leading to a transcriptional fusion (Fig. 1C). In contrast, gene trap constructs contain one or more splice acceptor sequences preceding the reporter

Biography: TANG Wei (1964-), Research associate, Forest Biotechnology Group, Department of Forestry, North Carolina State University, Raleigh, NC 27695-7247, USA.
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gene (Fig. 1B), which allow expression if insertion occurs in an intron. Splicing from the splice donor sites in the chromosomal gene to the splice acceptor sites in the reporter gene results in fusion of up-stream exon sequences to the reporter gene. In addition to transcriptional fusions, promoter trap and gene trap insertions can also create translational fusions, which may provide information about protein localization. Enhancer trap (Fig. 1D) (Springer 2000), the reporter gene is fused to a minimal promoter, typically containing a TATA box and transcription start site, that is unable to drive reporter gene expression alone but can be activated by neighboring enhancer elements.

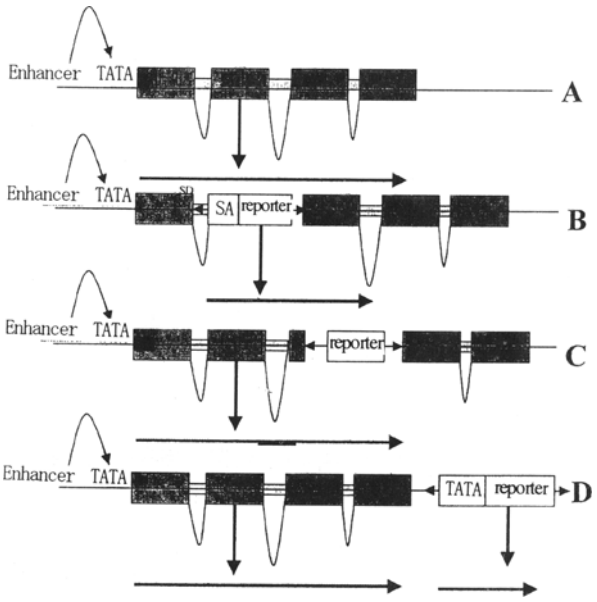

Fig. 1 Structure of gene trap, promoter trap, and enhancer trap tagging vectors.

(A) A generic chromosomal gene with exons (boxes) and introns (small boxes).

(B) Gene trap construct. The promoterless reporter gene contains splice acceptor (SA) sequences. Expression of the reporter gene occurs upon its insertion into an intron. Splicing from the chromosomal splice donor (SD) site to the SA sequence results in creation of a transcriptional fusion. Arrows in each panel represent the transcripts that are produced as a consequence of insertion.

(C) Promoter trap construct. The promoterless reporter gene can be expressed when insertion occurs in an exon so as to result in a transcriptional fusion.

(D) Enhancer trap construct. The minimal promoter of the reporter gene (TATA) is activated by a chromosomal enhancer element, resulting in ex-pression of the reporter gene.

Each type of reporter gene construct has its own advantages. Because enhancer traps do not have the same constraints on expression as promoter and gene traps, which must insert within a gene and in the correct orientation, enhancer trap insertions lead to a higher frequency of reporter gene expression. However, expressed promoter or gene traps are more likely to cause gene disruption than are ex-pressed enhancer traps. Also, because enhancers can activate gene expression at considerable distances, the genes controlling reporter gene expression may be more easily identified in promoter or gene trap ins ertions than in enhancer trap insertions.

Insertion Vehicle

T-DNAs.

T-DNAs is one of the two main alternatives for delivery of gene trap, promoter trap, and enhancer trap reporters into plant genomes. T-DNA–mediated transformation is one of the best ways to generate transgenic plants. In plants, the use of T-DNAs allows large collections of independent insertions to be quickly generated. Because T-DNAs are not known to insert with site specificity, it should be possible to saturate the genome with T-DNA insertions (Azpiroz-Leehan and Feldmann 1997). Recently, large T-DNA collections have been generated in Arabidopsis (Feldmann and Marks 1987; Bouchez *et al.*; 1993; Campisi *et al.*; 1999; Krysan *et al.*; 1999; Weigel *et al.* 2000). However, multiple T-DNA insertions often occur in a single plant, both in multiple copies per locus and in multiple loci (Bechtold *et al.* 1993; Lindsey *et al.* 1993). This multiplicity is a potential problem when delivering enhancer or gene trap reporter genes, because multiple insertions may complicate interpretation of expression patterns. In addition, genomic T-DNA insertions are generally stable, so that remobilization is not readily possible, as it is for transposable elements. T-DNA vectors have been used by a number of different groups to deliver gene trap constructs into plants. Enhancer, promoter, and gene trap reporter genes have been used, and expression of reporter genes has been efficient with the reporter gene positioned at either the left (Lindsey *et al.* 1993) or right (Campisi *et al.* 1999) border of the T-DNA.

Transposable elements

Transposable elements is another alternatives for delivery of gene trap, promoter trap, and enhancer trap reporters into plant genomes. In plant species that do not have active, well-characterized transposable element systems, transposable elements are routinely used to perform insertional mutagenesis by T-DNA–mediated transformation introduced into the plant genome and subsequently mobilized (Osborne *et al.* 1991). Insertional mutagenesis with transposable elements offers several advantages over

T-DNAs. The first is transposable element insertions can be selectively dependent upon expression of transposase and remobilization can generate germinal revertants so as to verify that a phenotype is indeed caused by insertion of the transposon. Second is somatic revertant sectors can also be generated for clonal analysis, which is useful for studying the cell-autonomous nature of the given gene product. Finally, because some eukaryotic elements preferentially transpose to closely linked locations (Greenblatt 1984), derivative alleles can be generated by remobilizing an element (Das and Martienssen 1995). The maize *Ac/Ds* and *En/Spm* transposable elements have been developed for use in heterologous species. The behavior of these elements has been extensively studied, and they have been modified for efficient transposition in plants such as tobacco, tomato, and Arabidopsis (Osborne and Baker 1995). At present, the *Ac/Ds* system has been used for enhancer or gene trap systems (Sundaresan *et al.* 1995). The advantage of *Ac/Ds* system used for gene trap tagging in plant is its low copy number (Bancroft *et al.* 1992). In maize, *Ac/Ds* elements demonstrate a marked preference for transposition to genetically linked locations in Arabidopsis and other plants (Dooner and Belachew, 1989; Jones *et al.* 1990; Dooner *et al.* 1991; Osborne et al. 1991; Bancroft and Dean 1993; Carroll *et al.* 1995; Machida *et al.* 1997).

Application of reporter gene

â-*Glucuronidase*

The bacterial â-Glucuronidase *(uid*A) gene is a commonly used reporter gene in genetic transformation of higher plants. The â-Glucuronidase protein (GUS) is quite stable, and it retains activity when it is fused to other proteins (Jefferson *et al.* 1987). GUS activity can be detected by histochemical staining using a variety of substrates that are commercially available, although most of the substrates are expensive and the histochemical stains are destructive, and GUS assays cannot be performed on live tissue. Detection of GUS activity is very sensitive (Jefferson *et al.* 1987; Lindsey *et al.* 1993), and activity can even be detected in single cells. Although it is difficult to conduct GUS staining for large plants or for large-scale screens on many plants, it is widely and successfully applied to Arabidopsis because this plant species is quite small and individuals can be stained in small volumes of substrate solution.

Green fluorescent protein

The green fluorescent protein (GFP) which is isolated from jellyfish has recently been used as a reporter gene in plants. Because GFP is fluorescent, it can be directly detected by illumination and no substrate is required. Detection of GFP activity is also nondestructive. It can be performed in live cells and can be monitored over time. Recently, different versions of GFP has been made for obtaining GFP proteins that fluoresce efficiently in Arabidopsis (Haseloff and Amos 1995; Siemering *et al.* 1996; Haseloff *et al.* 1997), and these modifications have improved the sensitivity of GFP (Haseloff *et al.* 1997), making it a useful reporter for gene trap systems.

Lc transcription factors

A member of the maize *R* gene family of Myc-like transcription factors, Lc transcription factors, it regulates anthocyanin biosynthesis and is particularly promising as a reporter gene. Expression of *Lc* in heterologous plants leads to anthocyanin accumulation (Lloyd *et al.* 1992; Goldsbrough *et al.* 1996). Therefore, its detection does not require expensive substrate and is nondestructive. This type of visual reporter is especially attractive for screens of large, field-grown plants, and for which illumination for GFP detection might be impractical.

Gene trap tagging and gene identification for plant development

T-DNA-mediated gene trap tagging

The first generation gene trap systems in plants were designed to determine how frequently T-DNA insertions integrated into genes. Early experiments were performed by transforming tobacco protoplasts with a T-DNA containing a promoterless antibiotic resistance gene adjacent to one border (André *et al.* 1986; Teeri *et al.* 1986). Recovery of a transformed plant relied on the generation of a gene fusion that led to expression of the antibiotic resistance gene. Therefore, this approach was mainly applied to detection of gene fusions that were expressed in regenerating tissues. Further modifications of the experimental approach incorporated a second selectable marker in the T-DNA, so that transformed plants could be regenerated and subsequently screened for expression of the promoterless antibiotic resistance gene (Koncz *et al.* 1989; Herman *et al.* 1990). The next step was inclusion of the â-glucuronidase *(gusA* or *uid*A) reporter gene that could be easily visualized by histochemical staining (Fobert *et al.* 1991; Kertbundit *et al.* 1991; Topping *et al.* 1991). This advance allowed spatial and temporal expression patterns to be visualized. Constructs that contained either a promoterless *gusA* gene or a *gusA* gene driven by a minimal promoter were used. More recently, transposable elements have been used to deliver enhancer or gene traps into plant genomes (Fedoroff and Smith, 1993; Klimyuk *et al.* 1995; Sundaresan *et al.* 1995).

Ac/Ds transposable element mediated gene trap tagging

Ac/Ds transposable element system is a two-component system with autonomous (*Ac*) and nonautonomous (*Ds*) components. The *Ac* element encodes a transposase that binds to the terminal inverted repeat ends of both *Ac* and *Ds* elements and catalyze their transposition to new locations in the genome. *Ds* elements are most often derivatives of *Ac* that have lost the ability to produce a transposase but retain the terminal inverted repeats. The *Ac* transposase, when produced in *trans*, is able to recognize the ends of *Ds* elements and catalyze their movement to new chromosomal locations. A gene trap system that uses selection for transposition was developed at Cold Spring Harbor Laboratory (Sundaresan *et al.* 1995). This system uses the *Ac*/*Ds* transposable elements and a positive/negative selection for transposition. The *GUS* gene in the enhancer trap element (*Ds*E) is fused to a minimal promoter from the CaMV 35S gene. This region of the promoter has no detectable activity unless chromosomal enhancer elements are nearby (Benfey *et al.* 1989). The *GUS* gene in the gene trap element (*Ds*G) is promoterless and contains three SA sites in each of three reading frames, fused upstream of the initial ATG codon. This construct allows *G* expression via transcriptional and translational fusion if the *DsG* element inserts in an intron. Additionally, naturally occurring splice donor sites in the 39 end of the *Ds* element (Wessler *et al.* 1987; Nussaume *et al.* 1995) allow splicing and expression if insertion occurs in an exon. In reality, only two of the three SA sites appear to be used (Nussaume *et al.* 1995), but reporter gene expression in transposants occurs reasonably high efficiency (Sundaresan *et al.* 1995). Each *Ds* element was subcloned into a binary T-DNA vector for introduction into Arabidopsis by *Agrobacterium*-mediated transformation. The counter selectable *iaaH* gene driven by the ubiquitously expressed 29 promoter, was included each T-DNA, thereby allowing both the *Ac* and the donor *Ds* loci to be selected against after mobilization (Springer 2000).

Identification of genes and markers

The most exciting use of gene trap tagging should be in the identification of genes with specific patterns of expression that are differentially regulated. Novel genes are likely to be found in any gene trap screen. Screens have in fact been successful in identifying genes specifically express in lateral roots (Malamy and Benfey 1997), developing embryos (Topping *et al.* 1994; Topping and Lindsey, 1997), and shoot apices (Springer et al. 1995). Conditional screens have also been performed to identify genes regulated by nematode infections (Barthels *et al.* 1997; Favery et al. 1998). At present, although only a few plant genes have been cloned and characterized using gene trap tagging, these experimental systems provide examples of identification of both essential and redundant genes. One specific example is the *PROLIFERA* (*PRL*) gene that was identified as a gene trap *DsG* insertion that showed GUS activity in dividing cells. *PRL* encodes a protein that is related to MCM7 (Springer *et al.* 1995), a member of the MCM gene family found in all eukaryotes and required for the initiation of DNA replication (Kearsey and Labib 1998). Expression in dividing cells is consistent with this predicted role in cell division. Disruption of *PRL* by the *DsG* element led to megagametophyte embryo lethality. Arrest of both megagametophytes and embryos occurred at variable stages of development (Springer *et al.* 1995, 2000). There are many embryo lethal mutations in Arabidopsis that have variable phenotypes, which makes it difficult to determine the cause of lethality (Meinke 1991). However, the *GUS* expression pattern in dividing cells suggested a role in cell division before the gene was cloned. In addition, an equally important use of gene traps is to identify tissue or cell specific expression patterns. Such expression patterns can then be used as markers to identify particular cells or tissues. Markers are useful tools for developmental analysis, although relatively few have been described in plants. Markers that are expressed in distinct patterns during development can be used to examine normal patterns of development and to characterize mutant phenotypes. Specifically, markers are useful for determining when changes in cell fate occur during morphogenesis and for following cell line ages during development. Molecular markers are particularly effective tools for analysis of mutant phenotypes. Because developmental mutations often disrupt cellular identity, it can be difficult to determine which specific defects are caused by a gene disruption. The availability of a collection of different cell type specific markers is very useful for determining cellular identity in mutant tissues. Markers are also useful for identifying very early alterations in developmental pattern. It is sometimes possible to trace a mutant phenotype to a defect that occurs at an early stage in development, perhaps before deviation from normal morphology or anatomy can be detected. Gene traps in plants have been used quite extensively in this capacity. Gene trap patterns have been used to study epidermal patterning (Berger *et al.* 1998), lateral root initiation (Smith and Fedoroff, 1995; Malamy and Benfey, 1997), leaf development (Tsukaya and Uchimiya 1997), flower development (Roe *et al.* 1997; Liljegren *et al.* 2000), and embryogenesis (Topping *et al.* 1994; Topping and Lindsey 1997; Willemsen *et al.* 1998).

Therefore, even in the absence of prior molecular information, gene trap expression patterns can be enormously useful.

Promoter trap tagging and promoter identification

Promoter trap contain a promoterless reporter gene so that expression can occur only when the insertion is within a transcriptional unit and in the correct orientation. Expression of a promoter trap reporter gene requires that it be inserted into an exon, leading to a transcriptional fusion. Promoter trap taggings allow for the identification of promoters that drive specific expression. Once identified, specific promoters can be used to drive the ectopic expression of experimental genes to examine patterns of development. An example of this was demonstrated recently by Tsugeki and Fedoroff (1999). The authors isolated a root cap specific promoter in an enhancer trap line. This promoter was then used to drive expression of a diphtheriatoxin gene *(DT*-A) to examine the developmental consequences of ablating root cap cells. An elegant modification of this approach makes use of the yeast transcriptional activator *GAL4* as a reporter gene. The GAL4 system was first used in *Drosophila* (Brand and Perrimon 1993) and has been adapted by Jim Haseloff for use in Arabidopsis (Haseloff et al. 1997). In this system, a T-DNA containing a modified yeast *GAL4* gene *(GAL4-VP16)* fused to the minimal CaMV 35S promoter and a modified *GFP (mGFP)* gene driven by the *GAL4* upstream activating sequence (UAS) are transformed into Arabidopsis. When *GAL4-VP16* is positioned under control of a chromosomal enhancer, expression can be visualized by GFP fluorescence, because GAL4 activates *GFP* expression from the UAS. A battery of enhancer trap lines expressing this GAL4-VP16 fusion in different patterns has been generated. This system can then be used to express a gene of interest ectopically in many different patterns upon fusion to UAS elements. Genetic crosses are used to bring the ectopic gene under control of the *GAL4-VP16* activator, expressed in a particular pattern that is visualized by GFP. This system can also be used to genetically ablate specific cells or morphological regions by expressing a toxin gene in the same way

Enhancer trap tagging and enhancer element identification

For enhancer trap tagging, the reporter gene is fused to a minimal promoter, typically containing a TATA box and transcription start site, which is unable to drive reporter gene expression alone but can be activated by neighboring enhancer elements. An enhancer trap *DsE* insertion was identified in the *AGL8* gene (Gu *et al.* 1998), which had previously been described as a member of a large family of MADS box genes related to *AGAMOUS* (Mandel and Yanofsky 1995). The *DsE* insertion in the 59 untranslated leader of *AGL8* caused a loss-of-function mutation that resulted in a failure of the silique to elongate after fertilization. This failure caused the developing silique to be shortened and crowded with seeds. *AGL8* was therefore renamed *FRUITFULL (FUL)* to reflect this phenotype (Gu *et al.* 1998). The defect in silique elongation in *ful* mutants was consistent with its expression pattern in the carpel valves. However, *FUL* was also expressed in the shoot apical meristem and up regulated in meristems undergoing a transition to flowering, suggesting a possible role in the reproductive tra..sition. Indeed, *ful* mutants showed as light delay in flowering time (Ferrándiz *et al.* 2000). However, when combined with mutations in related MADS box genes *APETALA1 (AP*1) and *CAULIFLOWER (CA*L), the *ful* mutation causes a complete failure to flower (Ferrándiz *et al.* 2000). Thus, *FUL* appears to act redundantly with *AP1* and *CAL* to promote the transition to flowering (Ferrándiz *et al.* 2000). This function was suggested by the expression pattern and was only uncovered by analysis of the triple mutant.

Genomics database resources

Because the Arabidopsis genome will soon be completely sequenced (Meinke *et al.* 1998), there is a high probability that even a small region of flanking sequence is sufficient to identify a corresponding genomic sequence. Such identification immediately yields the map position of the corresponding *Ds* insertion. Furthermore, because the genome sequence is being systematically annotated, predicted genes within the region of the insertion can also be identified, which allows for the identification of candidate genes driving reporter gene expression. A variety of gene trap lines exist that contain single *Ds* element insertions in the genome (Sundaresan *et al.* 1995; Martienssen, 1998; Parinov *et al.* 1999). To identify the gene that controls reporter gene expression, one can use various polymerase chain reaction (PCR) techniques to isolate genomic DNA flanking the insertions (Martienssen 1998). Thermal asymmetric interlaced PCR is particularly effective for amplifying flanking DNA sequences (Liu *et al.* 1995;Tsugeki *et al.* 1996). Once a small region of flanking sequence is known, then database searches can be performed to identify corresponding genomic DNA sequences and candidate genes. In the case of gene trap insertions (which result in transcriptional fusions), 59 RACE (for ran-

dom amplification of cDNA ends)–PCR can also be used to amplify exon sequence upstream of the insertion (Skarnes *et al.* 1992; Springer *et al.* 1995). This approach works well for genes that are relatively abundantly expressed. Because RACE-PCR yields exon sequences, candidate genes are directly identified, and cDNA libraries can be screened directly with PCR product probes. A number of groups are systematically amplifying and sequencing genomic DNA flanking random gene trap insertions in Arabidopsis. Databases containing this sequence information have been generated (Martienssen, 1998; Parinov *et al.* 1999). The eventual inclusion of expression data and phenotypic information will be an important component of these databases. This sequence information can be used for reverse genetic screens to identify insertions in previously cloned genes and members of gene families. Together, the sequence, expression, and phenotypic data will contribute to current efforts to determine the function of all genes in the Arabidopsis genome (Springer 2000).

Concluding remarks

Gene traps have proven to be highly useful tools in plant developmental biology. Their largest contribution has been in the generation of tissue and cell type specific markers, gene, promoter, and enhancer identification. In some cases, the genes regulating reporter gene expression have been surprisingly difficult to identify. Nonetheless, as these sequences of the Arabidopsis genome is completed, gene traps are likely to play an increasingly important role in the next phase of genomics: determining gene function. Compared to traditional gene identification methods, gene trap tagging has two main advantages. First, gene trap tagging can detect many genes that are functionally redundant, sharing overlapping functions with other genes that may or may not be related at the sequence level. Mutation of a functionally redundant gene is not likely to lead to an easily recognizable phenotype, because one or more other family members can provide the same function. Analysis of systematic gene knockouts has revealed that a significant percentage of yeast genes have no obvious phenotype when disrupted, despite testing under a wide range of growth conditions. Therefore, it is likely that disruption of many plant genes will not result in an easily identifiable phenotype; second, gene trap tagging can detect many genes that function at multiple stages of development. Mutations in these genes may lead to early lethality or may be highly pleiotropic, which can mask the role of a gene in a specific pathway. At present, although several different types of "trapping" systems have been developed, the major difference among such systems lies in the reporter gene construct that is used. In practical experimental research, the question that is often asked about gene traps is whether reporter gene expression patterns accurately mimic expression of endogenous genes and whether reporter gene patterns accurately reflects chromosomal gene expression. Therefore, widely extended research and support from government administrations and private companies are practically needed for gene trap tagging.

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